

Low TGFβ1 expression prevents and high expression exacerbates diabetic nephropathy in mice

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Nephropathy develops in many but not all patients with long-standing type 1 diabetes. Substantial efforts to identify genotypic differences explaining this differential susceptibility have been made, with limited success. Here, we show that the expression of the transforming growth factor β1 gene (*Tgfb1*) affects the development of diabetic nephropathy in mice. To do this we genetically varied *Tgfb1* expression in five steps, 10%, 60%, 100%, 150%, and 300% of normal, in mice with type 1 diabetes caused by the Akita mutation in the insulin gene (*Ins2*^{Akita}). Although plasma glucose levels were not affected by *Tgfb1* genotype, many features of diabetic nephropathy (mesangial expansion, elevated plasma creatinine and urea, decreased creatinine clearance and albuminuria) were progressively ameliorated as *Tgfb1* expression decreased and were progressively exacerbated when expression was increased. The diabetic 10% hypomorphs had comparable creatinine clearance and albumin excretion to wild-type mice and no harmful changes in renal morphology. The diabetic 300% hypermorphs had ~1/3 the creatinine clearance of wild-type mice, >20× their albumin excretion, ~3× thicker glomerular basement membranes and severe podocyte effacement, matching human diabetic nephropathy. Switching *Tgfb1* expression from low to high in the tubules of the hypomorphs increased their albumin excretion more than 10-fold but creatinine clearance remained high. Switching *Tgfb1* expression from low to high in the podocytes markedly decreased creatinine clearance, but minimally increased albumin excretion. Decreasing expression of *Tgfb1* could be a promising option for preventing loss of renal function in diabetes.

aldosterone | glomerular filtration rate | glomerulosclerosis | megalin | nephrin

Diabetes is the number one cause of end-stage renal disease in the United States and many other developed countries. However, despite having similar levels of blood glucose only 20–40% of all diabetic patients develop diabetic nephropathy. In diabetic nephropathy, increased expression of transforming growth factor β1 (TGFβ1) has been demonstrated to promote accumulation of extracellular matrix components (1), apoptosis (2), dedifferentiation of podocytes (3), and epithelial–mesenchymal transition of proximal tubules (4), all of which are thought to facilitate a decline in nephron number and renal function.

Tgfb1-null mice on a mixed genetic background show severe multiorgan inflammation with massive infiltration of lymphocytes and macrophages that culminates in death by 3–4 wk of age (5, 6). Their death effectively prevents determining whether absence of TGFβ1 influences the development of nephropathy. To overcome this problem and also to allow the study of the effects of above-normal TGFβ1, we have generated mice with five genetically graded levels of TGFβ1, and have made them diabetic with the *Ins2*^{Akita} mutation, which causes pancreatic beta-cell dysfunction and type 1 diabetes.

Here we show that the features characteristic of diabetic nephropathy are progressively minimized as *Tgfb1* expression is decreased below normal and are progressively exacerbated when expression is increased above normal.

Generation of Akita Diabetic Mice Having Five Genetically Different Levels of *Tgfb1* Expression

We recently described the generation of C57BL/6 mice having a low-expressing *Tgfb1* allele (*Tgfb1*^L), which can be switched to high expressing form (*Tgfb1*^H) by exposure to Cre recombinase, and the combination of these low and high expressing alleles with the wild-type allele (*Tgfb1*⁺) to produce mice having *Tgfb1* mRNA expression graded in five steps from 10% to 300% normal (7). We have now crossbred these mice with mice having the Akita mutation in the *Ins2* gene (8) to generate type 1 diabetic mice with different TGFβ1 levels. Male C57BL/6 Akita diabetic mice with the following five genotypes were studied: *Tgfb1*^{L/L};*Ins2*^{Akita/+} (hereafter called L/L:A/+), *Tgfb1*^{L/+};*Ins2*^{Akita/+} (L/+A/+), *Tgfb1*^{+/+};*Ins2*^{Akita/+} (WT:A/+), *Tgfb1*^{H/+};*Ins2*^{Akita/+} (H/+A/+), and *Tgfb1*^{H/H};*Ins2*^{Akita/+} (H/H:A/+).

Fig. 1A shows that Akita diabetic mice with the five *Tgfb1* genotypes have a graded expression of *Tgfb1* mRNA in their kidneys, and that their plasma TGFβ1 levels have a similar gradation (Fig. 1B). They all have about three times the plasma concentration of glucose and about one third the plasma insulin concentration of wild-type nondiabetic C57BL/6 mice, indicative of type 1 diabetes. These plasma glucose and plasma insulin concentrations were not significantly affected by the *Tgfb1* genotype (Fig. 1C and D and *SI Appendix*, Fig. S8).

General Characteristics of Akita Diabetic Mice with Five Graded Expressions of *Tgfb1*

The body weights of the L/L:A/+ Akita diabetic mice were about 15% less than those of the Akita mice with the other *Tgfb1* genotypes (*SI Appendix*, Table S1), a result similar to our previous

Significance

About one third of patients with type 1 diabetes mellitus develop nephropathy, which often progresses to end-stage renal diseases. The present study demonstrates that below normal transforming growth factor (TGF) β1 expression ameliorates the nephropathy and decreased glomerular filtration rate resulting from long-standing type 1 diabetes, while above normal TGFβ1 expression makes both worse. Reducing TGFβ1 expression in the glomerulus is more important in avoiding the decrease in glomerular filtration rate than altering expression in the tubule, while expression in the tubule is more important in controlling interstitial fibrosis and albuminuria. Suppressing TGFβ1 action in the kidney as a whole, or specifically in podocytes, could be a promising option for treating/preventing the progressive deterioration of renal function in diabetes.

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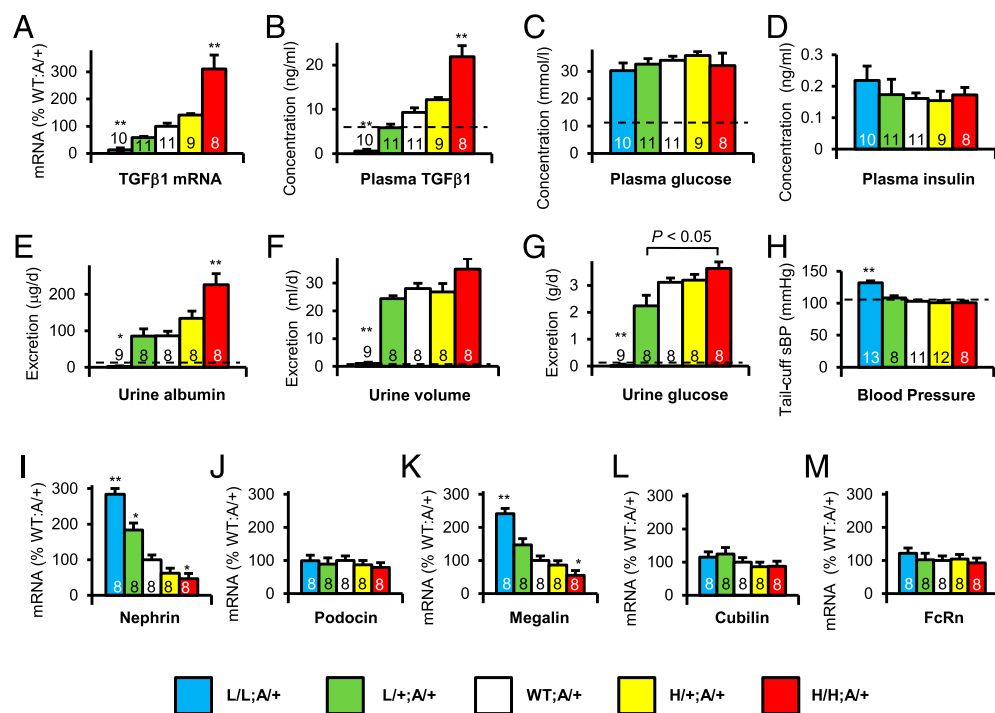


Fig. 1. Characterization at age 40 wk of Akita diabetic mice having five genetically determined levels of *Tgfb1* expression. (A) *Tgfb1* mRNA in the kidney. (B) Plasma concentration of TGFβ1. (C) Plasma glucose concentration. (D) Plasma insulin concentration. (E) Urinary albumin excretion. (F) Urine Volume. (G) Urine glucose excretion. (H) Systolic blood pressure. (I) Renal mRNA expression of Nephrin. (J) Podocin. (K) Megalin. (L) Cubilin. (M) Neonatal Fc receptor (FcRn). All of the mice were Akita diabetic. Bars are color coded to indicate *Tgfb1* and *Ins2* genotypes: blue (L/L:A/+), green (L/+A/+), white (WT:A/+), yellow (H/+A/+), red (H/H:A/+). *P < 0.05, **P < 0.01 vs. WT:A/+.

finding with nondiabetic L/L mice (7). The heart weights of the L/L:A/+ and H/H:A/+ mice were, respectively, ~10% and 20% lower than that of the mice with the other *Tgfb1* genotypes, but heart weight/body weight ratios did not differ significantly among all five genotypes (SI Appendix, Table S1). Heart rates were not significantly different (SI Appendix, Fig. S1). The kidney weight/body weight ratio was ~15% lower in the L/L:A/+ mice and ~30% higher in the H/H:A/+ mice in comparison with Akita diabetic mice with wild-type *Tgfb1* expression (SI Appendix, Table S1). Plasma cholesterol and plasma triglyceride concentrations were not affected by the *Tgfb1* genotypes.

Effects of *Tgfb1* on Urinary Excretion of Albumin, Water, and Glucose

Because nephropathy/renal failure in human patients is associated with long-term diabetes, the effects of graded expression of *Tgfb1* were studied in mature adult 40-wk-old C57BL/6 Akita diabetic mice. Using metabolic cages, we found that the L/L:A/+ diabetic mice, like the nondiabetic L/L mice, excreted very little amount of albumin (Fig. 1E and SI Appendix, Fig. S2). However, higher levels of TGFβ1 led to progressive increases in urinary albumin excretion (Fig. 1E), ranging from microalbuminuria in the L/+A/+ diabetic mice (~80 μg/day) to macroalbuminuria in the H/H:A/+ diabetic mice (~200 μg/day). The urine volumes of the L/L:A/+ Akita diabetic mice (Fig. 1F) were much reduced compared with the polyuric urine volumes of the other Akita diabetic mice (~1 mL/day versus ~30 mL/day). In addition to not having polyuria, the L/L Akita mice did not have glucosuria (Fig. 1G), even though they had about three times normal plasma glucose concentration and about one third normal plasma insulin concentration (Fig. 1C and D). Nondiabetic L/L mice also excreted very little amount of glucose (SI Appendix, Fig. S11). The L/L:A/+ mice had systolic blood pressures ~20 mmHg above normal (Fig. 1H). These unusual features are seen in nondiabetic L/L mice, caused by their having ~2× normal plasma aldosterone concentrations (7). Our L/L:A/+ diabetic hypomorphs also have plasma aldosterone concentrations about twice that of diabetic mice that are wild type at the *Tgfb1* locus (SI Appendix, Fig. S6). Thus, although the 10% hypomorphs developed additional features associated with their hyperaldosteronism, we conclude that higher-than-normal expression of *Tgfb1* in the Akita diabetic mice

caused increased albumin excretion, whereas lower than normal expression decreased the albuminuria.

Effects of *Tgfb1* on Expression of Genes Affecting Renal Function

To uncover factors affecting the nephropathy in our diabetic mice with graded expression of *Tgfb1*, we determined the expression in the kidney of mRNAs coding for proteins involved in renal function and albumin excretion (Fig. 1I–M). Nephrin (mouse gene: *Nphs1*) and podocin (*Nphs2*) were chosen because they are expressed in renal podocytes and have mutations that cause congenital nephrotic syndromes in humans (9–12). Megalin (*Lrp2*) and cubilin (*Cubn*), both expressed in the brush border of renal proximal tubules, were chosen because of their known contribution to the endocytosis of low molecular weight proteins and albumin (13–17). Neonatal Fc receptor (*Fcgrt*) was included because it is also expressed in the brush border but its primary effects are on the urinary excretion of immunoglobulins rather than of albumin (18, 19). The results show that genetic increases in the expression of *Tgfb1* in the Akita diabetic mice caused progressive decreases in the renal expression of nephrin and of megalin, ranging from ~250% normal in the L/L:A/+ mice to ~50% normal in the H/H:A/+ mice. Expression of cubilin and of the neonatal Fc receptor was unaffected. We conclude that progressive increases in *Tgfb1* expression from 10% to 300% normal are accompanied by progressive decreases in nephrin and megalin expression from ~250% to ~50% normal but without changes in the expression of podocin, cubilin, and the neonatal Fc receptor.

Excretory Function of Nondiabetic and Akita Diabetic Mice with Graded *Tgfb1* Expression

The effects of graded expression of *Tgfb1* on renal function were studied in mature adult 40-wk-old Akita diabetic mice and their nondiabetic counterparts. The results show that in the nondiabetic mice changes in *Tgfb1* expression had no significant effects on glomerular filtration rate (GFR) as judged by plasma levels of urea nitrogen and creatinine, and creatinine clearance (SI Appendix, Figs. S3–S5). However, in the Akita diabetic mice we found a highly significant inverse gradation in GFR as *Tgfb1* expression varied (Fig. 2A–C). Thus, GFR was greater than in

Nevertheless, our results indicate that this problem might be avoided, while still retaining efficacy, if the reduction of *Tgfb1* expression was only in the kidney.

In summary, we have studied the renal phenotype of mature Akita diabetic male mice having five genetically controlled levels of TGF β 1 and have demonstrated that below normal *Tgfb1* expression ameliorates the decreased GFR and nephropathy that result from long-standing type 1 diabetes, whereas above normal *Tgfb1* expression exacerbates these abnormalities. We have also shown that reducing *Tgfb1* expression in the glomerulus is more important in avoiding the decrease in GFR than altering expression in the tubule, whereas expression in the tubule is more important in controlling interstitial fibrosis and albuminuria. Suppressing TGF β 1 action in the kidney as a whole, or specifically in podocytes, could be a promising option for treating/preventing the progressive deterioration of renal function that leads to end-stage renal disease in many diabetic patients.

Materials and Methods

Animals. To study the effects of TGF β 1 on the phenotype in diabetes, we crossbred heterozygous and homozygous mice having hypomorphic (L) or hypermorphic (H) alleles for TGF β 1 on a C57BL/6 genetic background (7) with mice having heterozygous Akita mutation in the insulin 2 gene, which is an animal model of type 1 diabetes mellitus (Akita mice), on a C57BL/6 genetic background (The Jackson Laboratory) (8). Because the L allele can be converted into the H allele by Cre-loxP recombination, we used this property to generate mice with tissue-specific overexpression of TGF β 1 in the hypomorphs. To study the effects of proximal tubule-specific overexpression on the phenotype in the L/L Akita mice, we used the *Ggt1* promoter-driven Cre transgene (*Ggt1*-cre/ERT2; European Mouse Mutant Archive) (25), which is induced by tamoxifen injection (50 mg/kg/day IP in sesame oil for 5 d) at age 4 wk. To study the effects of podocyte-specific overexpression on the phenotype in the L/L Akita mice, we used the podocin (*Nphs2*) promoter-driven Cre transgene (*Nphs2*-cre; The Jackson Laboratory) (26). All mice were kept under the husbandry conditions in conformance with guidelines of University of North Carolina Institutional Animal Care and Use Committee.

Measurement of Biological Parameters. Plasma glucose levels were determined with the glucose oxidase method (Wako Chemical). Plasma insulin levels were determined with ELISA (Crystal Chem). Plasma urea nitrogen concentrations and plasma and urine electrolytes were determined with the Vitros 250 Chemistry system (Ortho-Clinical Diagnostics). Plasma total cholesterol (Wako) and triglyceride (Stanbio Laboratory) were measured with enzymatic colorimetric methods. Plasma creatinine levels were studied with liquid chromatography tandem mass spectrometry (LC-MS/MS) as described (27). Plasma TGF β 1 and aldosterone were studied with ELISA (Quantikine Mouse/Rat/Porcine/Canine TGF β 1 Immunoassay, R&D Systems; Aldosterone EIA kit, Enzo Life Sciences). Metabolic balance studies were performed using metabolic cages (Solo Mouse Metabolic Cage; Tecniplast).

Histology. After the inferior vena cava is cut, the left ventricle was punctured by a 23-gauge needle and perfused with PBS for 3 min and with 4% paraformaldehyde for 5 min. Thereafter, the tissues were dissected out and put in 4% paraformaldehyde at least 3 d. These were then paraffin embedded and sectioned. The stained sections were prepared by Center for Gastrointestinal Biology and Diseases Histology Core and imaged on an Olympus BX61 microscope. For electron microscopy, grids were prepared by Microscopy Services Laboratory and imaged on a Zeiss TEM 910 transmission electron microscope.

Blood Pressure and Pulse Rate Measurement. We measured blood pressure and pulse rate with the tail-cuff method (28).

Quantitative Reverse Transcription-PCR. Total RNA was extracted from different tissues and the mRNAs were assayed by quantitative reverse transcription-PCR as described (29). The primers and the probes used to measure the mRNAs are shown in *SI Appendix, Table S2*.

Statistical Analysis. Data are expressed as means \pm SEs. To compare groups, we used one-factor or two-factor ANOVA. Post hoc pairwise comparisons were performed by Tukey–Kramer Honestly Significance Differences test (JMP 9.0; SAS Institute).

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